

Heat-Stable Defatted Rice Bran Protein Extracted by Subcritical Water Extraction

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Abstract: Protein in heat stable defatted rice bran (HSDRB) was extracted with subcritical water at various temperatures (100–200 °C) and various extraction durations (0–30 min). The extracts were analyzed for protein and total carbohydrate contents, amino acid composition, molecular mass distribution and color parameters. The results showed that the highest yields of protein and amino acids were 50% and 48.6 mg/g HSDRB, respectively at 175 °C for 30 min. Under these conditions, the average hydrophobicity of amino acids and the essential amino acids content were the highest, reaching 1.84 kJ/mol and 14.8 mg/g HSDRB, respectively. The color parameters were significantly different at various temperatures. L^* values were negatively correlated with protein content while a^* values were positively correlated with protein content. The hydrophilic fraction of the extracts mainly contained low-molecular-mass substances.

Key words: heat-stable defatted rice bran (HSDRB); subcritical water extraction (SWE); protein; total carbohydrates; amino acids

亚临界水提取热稳定脱脂米糠蛋白

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摘 要: 利用亚临界水在不同温度 (100~200 °C) 和时间 (0~30 min) 条件下提取热稳定脱脂米糠 (heat stable defatted rice bran, HSDRB) 中的蛋白质。研究在不同提取条件下提取物中蛋白质含量、总糖含量、氨基酸组成、分子质量分布以及色差。结果表明: 在提取温度175 °C、提取时间30 min时, 提取物中蛋白质及氨基酸的含量最高, 分别为50%和48.6 mg/g HSDRB; 在此条件下, 氨基酸具有最高的疏水性 (1.8 kJ/mol) 以及最高的必需氨基酸含量 (14.8 mg/g HSDRB); 温度对提取物颜色具有显著影响, L^* 值与提取物中蛋白质含量呈负相关关系, 而 a^* 值与提取物中蛋白质含量呈正相关关系; 提取物中的疏水性成分主要为小分子物质。

关键词: 热稳定脱脂米糠; 亚临界水提取; 蛋白质; 总糖; 氨基酸

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Rice bran is a by-product of rice milling that has only become available in recent years due to the centralization of rice milling. China, Japan, Korea, India, and the U.S. produce food oil from heat stable rice bran, leaving large amounts of low value heat stable defatted rice bran (HSDRB). The protein content of rice bran is about 10%–15%, consisting of 37% water-soluble, 31% salt-soluble, 2% alcohol-soluble and 27% alkali-soluble storage proteins^[1]. The protein efficiency ratio (2.0–2.5) and lysine content were also high compared

with other cereal proteins. Rice bran protein, a good source of hypoallergenic protein and suitable ingredient for infant food formulation, has stimulated researchers to come up with greener, more efficient and economical extraction methods^[2]. Typically, wet alkaline aqueous solution has been used to extract vegetable proteins. The protein recovery by alkaline extraction from raw rice bran was ca. 50% but decreased to ca. 35% after the heat stabilization^[3]. At the experimental level, protein was also isolated from HSDRB by physical

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and/or enzymatic methods with yields up to 67%^[3-5]. However, there are some disadvantages of these methods, such as the production of lysinoalanine under alkaline conditions or the high cost of enzymes^[2,6].

Subcritical water extraction (SWE) has emerged as a more useful technique that is efficient, economical, environmentally friendly, safe, and fast^[7-8]. SWE utilizes water between 100 °C and 374 °C under high pressure to maintain water in liquid state (critical point of water is 74 °C at 22.4 MPa)^[9]. The increase in the solvency power of subcritical water can be attributed to the reduction in its dielectric constant (ϵ) and increase in its water ionization constant with increasing temperature^[10]. As the temperature of water increases from ambient to 250 °C, ϵ decreases from 80 to near 27, which is similar to that of ethanol ($\epsilon = 24$) and methanol ($\epsilon = 33$) at 25 °C^[11-12]. Concentrations of both hydronium and hydroxide ions in subcritical water are high. These ions can catalyze many chemical reactions, such as hydrolysis of peptide or glycosidic bonds without any catalyst in SWE^[6]. SWE conditions can result in the hydrolysis of proteins and carbohydrate polymers while the lowered dielectric constant can increase the solubility of hydrophobic peptides. Therefore, proteinaceous materials, such as rice bran^[9], black rice bran^[7], soybean meal^[13], fish meat^[14], silk fibroin^[15], and yeast cells^[16], can be hydrolyzed in subcritical water without additional catalysts. However, amino acids and sugars can undergo further decomposition and reactions, leading to undesirable color, flavor and odor. Viscosity and surface tension of subcritical water decrease at high temperatures, facilitating mass transfer and penetration of water into the matrix of particles, and consequently lead to enhanced extraction efficiency^[17]. Mass transfer of water may be important to the extraction of HSDRB since the residual fat content in bran makes protein less accessible to water under atmospheric conditions^[13]. Recent studies reported that different composition of protein and yields of amino acids under similar SWE conditions^[6,18]. Rice bran is rich in polysaccharides, which can be hydrolysed to water soluble sugars during SWE. Therefore, the purpose of this study is to investigate changes in contents of protein and total carbohydrates at different extraction duration and temperatures, their physical properties and effects on the color of extracts, in the hope of obtaining results that may contribute to the understanding of suitable reaction conditions and chemistry of rice protein extraction by SWE.

1 Materials and Methods

1.1 Materials and Chemicals

Granular HSDRB (ground in a hammer mill to pass through a 0.38 mm mesh screen and then stored at 4 °C) Xuzhou Oil Co., Jiangsu Province, China; Cytochrome C (12 500 D), aprotinin (6 500 D), bacitracin (1 450 D), tetrapeptide GGYR (451 D), and tripeptide GGG (189 D) Sigma Co., St. Louis, MO, USA.

1.2 Instruments and Equipment

GS batch reactor Weihai Zhengwei Chemical Machinery Co., Ltd., China; Auto Science AP-01P vacuum pump Tianjin Automatic Science Instrument Co., Ltd., China; UV-2800 UV-Vis Spectrophotometer Shanghai Sunny Hengping Scientific Instrument Co., Ltd., China; 600 reverse phase high-performance liquid chromatography (RP-HPLC) Waters Technologies (Shanghai) Limited; TSK gel 2000 SW column Tosoh, Tokyo, Japan; CR-400 Chroman Meter Konica Minolta Co., Japan.

1.3 Methods

1.3.1 Basic results of HSDRB

The contents of moisture, crude fat, crude protein, crude ash, and nitrogen-free soluble substances were measured according to AOAC methods^[19].

1.3.2 Subcritical water extraction of HSDRB protein

Distilled water was degassed to remove dissolved oxygen. A 500 mL stainless steel batch reactor was charged with 300 mL 10% (*m/V*) suspension of HSDRB. The reactor was heated using an electric heater to the desired temperature (100, 125, 150, 175, and 200 °C) while shaking at 400 r/min for 10–30 min. The pressure in the reactor, 101.35 to 1 580 kPa, was estimated from the reactor temperature. The time required to raise the temperature from ambient to 100, 125, 150, 175, and 200 °C were 20, 30, 37, 45, and 50 min, respectively. The heating time of the reaction was counted after the set reaction temperature was reached. Upon the set reaction time, the reactor was immediately cooled to room temperature by immersing in a cool water bath for approximately 3–10 min. The reaction products were separated into a solid residues and soluble products by filtration under vacuum. The extract was kept in refrigerator at 4 °C until further analysis.

1.3.3 Protein yield

Protein yield of the extract was determined by the AOAC method^[19] with a protein conversion factor of 5.95. The protein yield was calculated as follows:

$$\text{protein yield/\%} = \frac{\text{total content of extraction/g}}{10 \text{ g (weight of HSDRB)} \times \text{protein content of HSDRB}} \times 100 \quad (1)$$

1.3.4 Total carbohydrate content

The total carbohydrate content of SWE was determined by the modified phenol-sulfuric acid method at a wavelength of 490 nm by UV-Vis Spectrophotometer using water as blank^[20]. Briefly, approximately 1.0 mL 5% (*m/m*) aqueous phenol solution and 5.0 mL 18.4 mol/L H₂SO₄ were added to 2.0 mL of diluted extract or glucose solution and then well mixed. The mixture was left for 10 min at ambient temperature and then cooled in the 25 °C water bath for 10 min before reading.

1.3.5 Amino acid analysis

Amino acids in the extracts was analyzed using the method of Zhang et al.^[21]. Briefly, approximately 2.0 mL extract solution was subjected to acid hydrolysis using 2.0 mL of 6 mol/L HCl under nitrogen atmosphere for 24 h at 110 °C. The hydrolysate was washed into a 50 mL volumetric flask and made up to the mark with distilled water. The amino acids were subjected to RP-HPLC analysis after a pre-column derivatisation with *o*-phthaldialdehyde (OPA). Methionin and cysteine were determined separately by their oxidation products according to the performic acid procedure prior to hydrolysis in 6 mol/L HCl. Amino acid composition was reported as mg amino acid per g HSDRB. Amino acid score (AAS) for each sample was determined using the following formula^[22]:

$$\text{AAS} = \frac{\text{g of amino acid per 100 g of test protein}}{\text{g of amino acid per 100 g of reference protein}} \times 100 \quad (2)$$

The limiting amino acid was defined as the amino acid with the lowest AAS. Essential amino acid index (EAAI) was calculated using the following formula^[23]:

$$\text{EAAI} = \sqrt[n]{\frac{100a}{A} \times \frac{100b}{B} \times \dots \times \frac{100g}{G}} \quad (3)$$

where *a*–*g* refers to amino acid contents/(mg/g protein) in the sample protein; *A*–*G* were amino acid contents in egg protein/(mg/g protein); *n* refers to the number of amino acids (*n* = 7).

1.3.6 Color measurement

The color of the SWE was measured using a Chroman meter and the color space results were expressed in terms of CIE *L***a***b** (CIELAB) based on the method of Watchararuij et al.^[13].

1.3.7 Molecular weight distribution

Molecular weight distributions of protein extracted from rice bran were determined by gel permeation chromatography (GPC) using a RP-HPLC system. A TSK gel 2000 SW column (300 mm × 7.8 mm, 5 μm) was equilibrated with

45% acetonitrile (*V/V*) in the presence of 0.1% trifluoroacetic acid. The SWE extracts (100 μg/50 mL) were injected into the column at an eluent flow rate of 0.5 mL/min and monitored at 220 nm at ambient temperature. A molecular weight calibration curve was prepared from the average absorption time of the following standards: cytochrome C (12 500 D), aprotinin (6 500 D), bacitracin (1 450 D), tetrapeptide GGYR (451 D), and tripeptide GGG (189 D). With the help of elution time of calibration materials, the linear regression equation was obtained for the calculation of molecular weight.

$$\lg M_w = 6.73 - 0.217T \quad (4)$$

where *M_w* is the molecular weight of protein; *T* is the elution time/min.

1.4 Statistical analysis

All tests were done in triplicate and data were expressed as means ± standard deviation (SD). Differences among groups were determined by one-way ANOVA analysis of variance using the Minitab 15 statistical program (Minitab INC., State college, PA, USA). The significance was defined at the 95% confidence level. Correlations were determined by the SPSS 16.0 statistical program (IBM INC., New York, USA).

2 Results and Analysis

2.1 Basic results of HSDRB

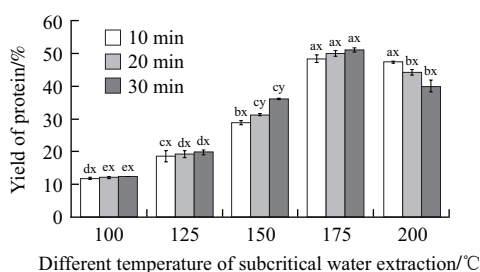
The HSDRB contains 9.7% moisture, 2.7% crude fat, 16.7% crude protein, 10.8% crude ash, and 60.2% nitrogen-free soluble substances.

2.2 Effects of extraction duration and temperatures on the protein yield

SWE efficiencies are usually evaluated by extraction duration, temperature, pressure, modifiers/additives, and static or dynamic operation mode^[24]. In this study modifiers were not used and therefore the extraction efficiency depended mainly on effects of extraction temperature and duration. The protein yields of the extracts were mainly dependent on the temperature in the range of 100–200 °C, as shown in Fig.1. The protein yield increased from 12% to 50% (ca. 85 mg/g HSDRB) with the increasing temperature from 100 °C to 175 °C. Complete extraction of protein was reported by Sereewatthanawut et al.^[6]. Wiboonsirikul et al.^[18] reported the highest protein yield of ca. 120 mg/g defatted rice bran, which was comparable to this study. Wiboonsirikul et al.^[9] also reported that the protein yield of defatted rice bran increased with increasing temperature up to 200 °C and significantly (*P* < 0.05) decreased at

250 °C. However, the protein yields at 20 min and 30 min at 200 °C were decreased by 14% and 28% compared to 175 °C in this study. The increased extraction yield with the increased temperatures could be attributed to the decrease in the dielectric constant of water, from ca. 80 to 40, and the increased hydrolytic activity caused by the increase in water ionization constant^[13,24-25]. At the highest temperature, the recovery of protein decreased, which might be attributed to the degradation of some thermal labile compounds after liberated from the source matrix^[26].

The protein yields could also be linked to the increase in hydrolysis duration. Nevertheless, the reaction duration did not significantly ($P > 0.05$) affect the protein yield under treatments with different temperature except 150 °C. In this study, the protein yields at 200 °C decreased by 16% from 10 min to 30 min. Wiboonsirikul et al.^[18] also reported that the protein yield at 200 °C and 260 °C decreased by about 25% and 40% from 0 min to 30 min and then remained relatively constant from 40 min to 120 min. The decreased yield with longer extraction duration may be due to the greater dissociation of water to H^+ and OH^- at higher temperatures^[13]. These results were supported by chromatographic analysis of the extracts. However, Sereewatthanawut et al.^[6] reported different results, where the protein yields at 200 °C increased from 50 mg/g rice bran protein to ca. 225 mg/g rice bran protein when the extraction time increased from 10 min to 30 min. In Sereewatthanawut's study, the extraction time did not significantly ($P > 0.05$) affect the protein yield at lower temperatures (100–160 °C). These differences in the effect of extraction duration might be due to differences of rice variety, milling, oil extraction process conditions, and static or dynamic SWE conditions.



Different letters [(a, b, c, d, and e) and (x, y, and z)] indicated significant difference unless otherwise stated.

Fig.1 Effects of reaction duration and temperature on the protein yields of the extracts

2.3 Effects of treatment duration and temperatures on total carbohydrate content

Total carbohydrate contents of the extracts from starch

hydrolysate and fiber produced at various temperatures and extraction durations were shown in Fig.2. The hydrolysis duration significantly ($P < 0.05$) affected the total carbohydrate content at various temperatures. The total carbohydrate content increased from ca. 120 mg/g HSDRB to ca. 200 mg/g HSDRB as the treatment temperature increased from 100 °C to 150 °C and then slightly ($P > 0.05$) decreased at 175 °C. It was then significantly ($P < 0.05$) decreased at 200 °C. At and above 175 °C, carbohydrate oligomers and monomers can be decomposed to non-glucose aqueous products, oil, char and gases^[13,27], resulting in the observed lower total carbohydrate content. Unlike the protein yield, the hydrolysis duration significantly ($P < 0.05$) affected the total carbohydrate content at various temperatures. For treatments at 100, 150 °C, and 200 °C, the highest total carbohydrate content was obtained at 10 min. For the treatments at 125 °C and 175 °C, the highest total carbohydrate content was obtained at 20 min. The total carbohydrate contents were the lowest at 30 min for all the treatment temperatures except 125 °C. Longer treatment duration did not always give the positive results, which were in agreement with results reported by other researchers^[3]. Therefore, a lower temperature of 150 °C and short duration (10 min) were suitable to generate a higher carbohydrate content.

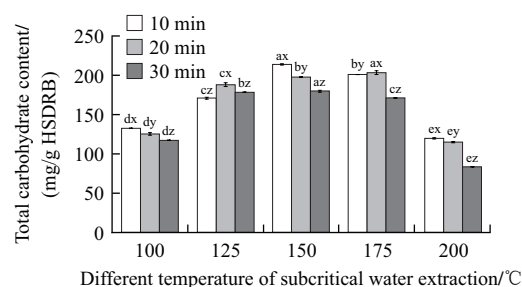


Fig.2 Effects of reaction duration and temperature on total carbohydrate contents of the extracts

2.4 Amino acid composition of extracts

Hydrolysis of proteins to amino acids increases with increasing temperature due to higher thermal energy, ionization of water, and enthalpy of solution of insoluble components. Since the highest protein extraction efficiency was obtained at 30 min, the amino acid composition of the extracts at various treatment temperatures for 30 min was determined (Table 1). The yields of 17 amino acids were found to increase from 11.4 mg/g HSDRB to 48.6 mg/g HSDRB with the increase of the treatment temperatures (100–175 °C), but decreased to 41.2 mg/g HSDRB at 200 °C. Our results were in agreement with those of Sereewatthanawut et al.^[6], where amino acids yields of rice bran decreased from 8 mg/g rice

bran to 5 mg/g rice bran as the temperature increased from 200 °C to 220 °C. This might be due to the decomposition of amino acids at higher reaction temperature into low-molecular-weight carboxylic acids and gaseous products at high reaction temperature^[16].

Amino acid analysis also showed that the contents of the most hydrophobic amino acids (Ile, Tyr, Phe, Leu, Pro, and Val) increased from 2.6 mg/g HSDRB to 17.6 mg/g HSDRB as the reaction temperature increased from 100 °C to 175 °C. Furthermore, the ratio of hydrophobic amino acids contents to total amino acids contents at 175 °C increased about 56% compared with the ratio at 100 °C.

In order to further evaluate the effects of different treatment temperatures on the amino acid composition, the average hydrophobicities of amino acids were calculated from the hydrophobic values for each amino acid^[28]. The average hydrophobicities increased from 0.35 to 1.84 as the temperature increased from 100 °C to 175 °C (Table 1). The relative dielectric constant decreased with the increasing temperature from 100 °C to 175 °C and there was a negative correlation between the average hydrophobicities and the relative dielectric constant ($r = -0.915$, $P < 0.05$). Furthermore, the yields of seven of the EAA substantially increased from 2.8 mg/g HSDRB to 14.8 mg/g HSDRB as the extraction temperature increased from 100 °C to 175 °C but slightly ($P > 0.05$) decreased (14.2 mg/g HSDRB) at 200 °C.

Table 1 Amino acid contents of HSDRB extracted using SWE at different extraction temperatures for 30 min

Amino acid	100 °C	125 °C	150 °C	175 °C	200 °C
Asp	1.92	3.21	5.98	4.51	2.59
Glu	2.31	3.53	7.86	10.44	12.56
Ser	0.52	0.98	2.05	2.61	1.57
His	0.35	0.58	1.07	1.36	1.05
Gly	0.95	1.66	2.77	3.91	2.93
Thr	0.50	0.89	1.72	2.21	1.30
Arg	0.80	1.56	2.51	0.58	0.45
Ala	0.82	1.57	2.96	3.97	3.60
Tyr	0.20	0.54	1.12	2.88	2.16
Cys	0.09	0.13	0.15	0.10	0.10
Val	0.53	0.80	1.92	2.97	2.73
Met	0.13	0.16	0.55	0.68	0.49
Phe	0.48	0.64	1.74	2.71	2.16
Ile	0.24	0.43	1.15	1.74	1.59
Leu	0.52	1.02	2.53	3.83	3.15
Lys	0.45	0.80	1.26	0.68	0.31
Pro	0.61	1.07	2.55	3.45	2.48
Totals	11.40	19.56	39.89	48.64	41.21
Average hydrophobic value/(kJ/mol)	0.35	0.61	1.35	1.84	1.52

Lysine was found to be the limiting amino acid in most cereals but was abundant in rice bran protein^[2]. However, the AAS of lysine was lower at all temperatures (Table 2) compared with the Food and Agricultural Organization/World Health Organization (FAO/WHO) requirement level^[29]. The AASs of other amino acids extracted at 175 °C and 200 °C were as high as or even higher than those required by FAO/WHO (Table 2). Another measure of protein quality is EAAI and the highest EAAI value was obtained at 150 °C. These results indicated that yields of EAA with high nutrition values may be increased under appropriate conditions using SWE.

Table 2 AAS and EAAI of HSDRB extracted using SWE for 30 min at different temperatures

	100 °C	125 °C	150 °C	175 °C	200 °C	FAO/WHO*
His	88	93	93	84	82	—
Ile	42	47	68	73	85	40
Leu	38	47	64	68	72	70
Lys	37	42	36	14	8	55
Met+Cys	44	31	47	37	36	35
Phe+Tyr	52	57	75	104	103	60
Thr	17	29	49	69	52	40
EAAI	33.9	35.6	42.8	37.2	33.8	

Note: *.data from reference [29].

2.5 Color parameter of extracts

The intensity of color during SWE is an indicator of chemical reaction and development of flavor compounds. The color of hydrolysates changed from white to yellow and further to brown with increasing temperature, which was expressed by L^* ($L^* = 0$ is black and $L^* = 100$ indicates diffuse white), a^* , and b^* CIELAB color space^[13]. The results of the color measurements were shown in Fig.3. Temperature significantly ($P < 0.05$) affected L^* , a^* , and b^* values of the hydrolysates. Interestingly, L^* value was highest at 125 °C and then declined, indicating an initial loss of chromophores in the visible range. The highest a^* value and lowest b^* values were obtained at 200 °C and 125 °C, respectively, for different hydrolysis duration. The increase of a^* values at 175 °C and 200 °C indicated that higher temperatures resulted in the formation of increased amounts of highly conjugated compounds^[30]. We also found that L^* values were negatively correlated with a^* values for 10, 20, and 30 min ($r = -0.881$, $P < 0.05$, $r = -0.953$, $P < 0.05$, and $r = -0.844$, $P = 0.073$, respectively), which was in agreement with the formation of more conjugated compounds. The formation of colored compounds is probably due to the Maillard reaction, a non-enzymatic browning process between reducing sugars and free amino acids^[13]. The negative correlations between the protein yields and L^* values for different

hydrolysis durations (10, 20, and 30 min) were observed ($r = -0.89$, $P = 0.098$, $r = -0.936$, $P < 0.05$, and $r = -0.94$, $P < 0.05$, respectively). And the protein yields for different hydrolysis duration had positive correlations with a^* values ($r = 0.94$, $P < 0.05$, $r = 0.889$, $P < 0.05$, and $r = 0.858$, $P = 0.063$, respectively). However, correlations between total carbohydrate contents and L^* , a^* , and b^* color space values were not observed, which indicated that the carbohydrate content was not the limiting factor for the color formation in this study.

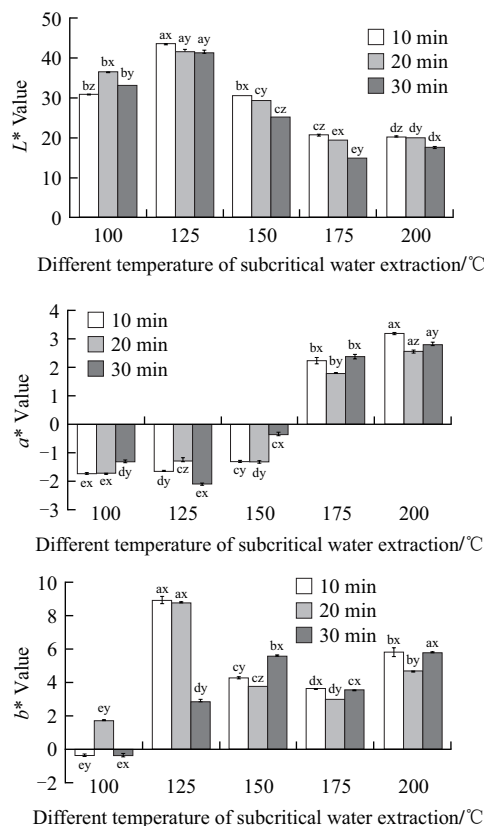
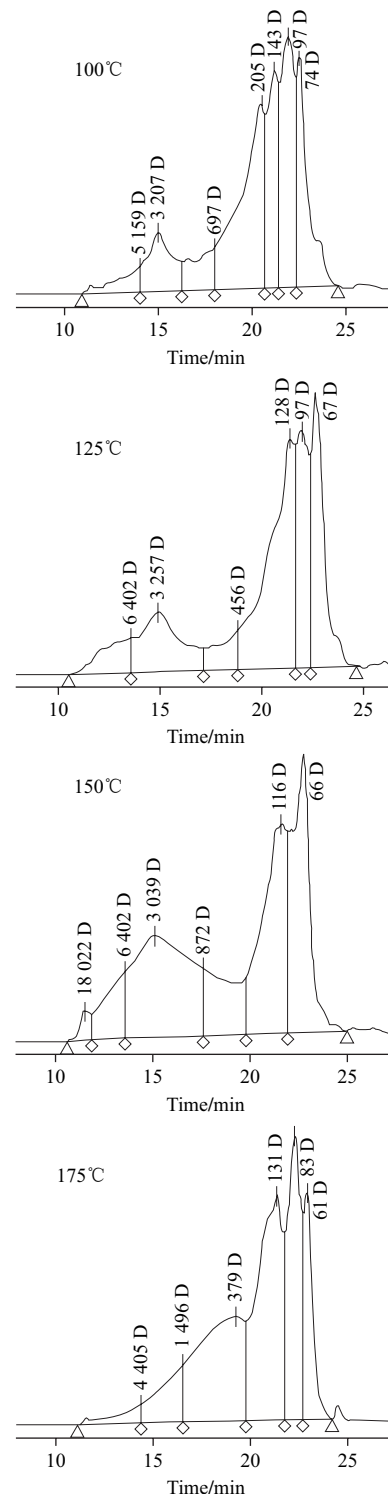


Fig.3 Color parameters of solution products measured using colorimeter

2.6 Molecular sizes of the extracts

Eluants with molecular masses of less or more than 10 kD were conveniently designated as the low and high-molecular-mass substances, respectively, following the convention of Wiboonsirikul et al.^[18]. R^2 of the calibration curve was 0.997. Size-exclusion chromatograms of the hydrolysates extracted at 100–200 °C for 30 min are shown in Fig.4. No high-molecular-mass substances were in the extracts at 100 °C and Wiboonsirikul et al.^[18] suggested that this was due to their insolubility in water at 100 °C. At higher temperatures, 175–200 °C, high-molar-mass substances were also absent and about 90% of the extracted protein had molecular weights of less than 700 D presumably because of thermal degradation of proteins into smaller molecules

of soluble protein or amino acids. Wiboonsirikul et al.^[18] observed the greatest amounts of high-molecular-mass substances at 200 °C but their extraction duration was much shorter (5 min). At 200 °C for 30 min, the SWE hydrolysates were estimated to be in the range of 500–8 000 D which is much higher than what we observed under the same conditions.



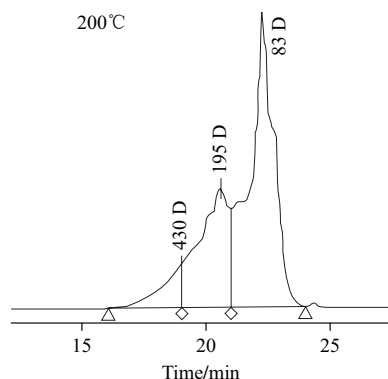


Fig.4 Chromatograms showing the molecular weight distributions of the rice bran extracts treated at different temperatures for 30 min

3 Conclusion

This study demonstrated that subcritical water could be used to hydrolyze HSDRB to obtain protein and amino acids. The treatment temperature, instead of hydrolysis duration showed significant effects on the yields of protein and amino acids. At the same time, higher total carbohydrate content was also obtained. However, we obtained different results from other recent studies although the conditions were similar. The results suggest that SWE conditions are extremely sensitive to minor differences in components or conditions.

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